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Kinetic Studies of Dogfish Liver Glutamate Dehydrogenase with Diphosphopyridine Nucleotide and the Effect of Added Salts*

(Received for publication, December 23, 1966)

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SUMMARY

Kinetic studies have been performed with crystalline dogfish liver glutamate dehydrogenase with the diphosphopyridine nucleotides as cofactors. The kinetic constants for the various substrates have been determined. In its sensitivity to guanosine 5′-triphosphate, adenosine 5′-diphosphate, and excess reduced diphosphopyridine nucleotide the enzyme is similar to that derived from other vertebrates. The extent of inhibition of the initial rate of the aminating reaction by excess reduced diphosphopyridine nucleotide was reduced by the presence of increased ammonium chloride in the reaction mixture. The effect is attributable to the chloride ion. A number of anions have been tested, and the order of their effectiveness in preventing inhibition by excess reduced coenzyme followed the Hofmeister series: ClO_4^- > SCN^- > I^- > Br^- > Cl^- > F^-. Peak effectiveness was observed at comparatively low concentrations of salts, varying from 0.045 M ClO_4^- to 0.25 M Cl^-. Further increase in salt concentration caused a progressive decrease in the rate of reaction. Concentrations of salts that were effective in preventing inhibition by excess reduced diphosphopyridine nucleotide were observed to depress activity at noninhibitory levels of coenzyme. Added salt does not affect the calculated maximum velocity of the reaction but does increase the apparent K_m for the diphosphopyridine nucleotide and effectively prevents inhibition at high concentrations of the cofactor. The presence of added salt minimized the regulatory influence of the nucleotides guanosine 5′-triphosphate and adenosine 5′-diphosphate.

There is considerable published evidence supporting the hypothesis that variations in the activity of beef liver glutamate dehydrogenase, in the presence of inhibitors and activators, are related to variations in the "associability" and "dissociability" of the enzyme in ultracentrifugal studies (1). There is some evidence that the same behavior may be characteristic of all glutamate dehydrogenases of animal origin (2–4). In view of the finding that dogfish liver glutamate dehydrogenase appeared to be incapable of any significant alteration in its sedimentation rates, as studied in the ultracentrifuge (5), it seemed logical to explore further some of its kinetic characteristics.

In particular, the effects of guanosine 5′-triphosphate, an inhibitor, adenosine 5′-diphosphate, an activator, and excess reduced diphosphopyridine nucleotide, which also causes inhibition, have been examined in this study. The strong qualitative similarities between the kinetic behavior of the dogfish enzyme and other animal glutamate dehydrogenases that have been described (5) serve to emphasize the lack of any correlation, in the case of the dogfish enzyme, between kinetic behavior and sedimentation characteristics.

In addition, ammonium chloride and ammonium acetate have been compared as substrates for glutamate dehydrogenase, and investigations have been carried out on the effects of added salts on the kinetic parameters of the enzymatic reaction with the diphosphopyridine nucleotides as cofactors.

MATERIALS

Glutamate was purchased as the monosodium salt from Nutritional Biochemicals, and α-ketoglutarate (Grade A) from Calbiochem. Nucleotides were obtained from the P-L Laboratories or Sigma. All other chemicals used were reagent grade from Fisher. Ammonium acetate, which is very deliquescent, was kept in a vacuum desiccator and weighed out quickly when needed.

The preparation of enzymes used in these studies has been previously described (5).

Investigations of the dogfish enzyme were greatly facilitated by the assistance of the New England Enzyme Center in the preparation of a large quantity of acetone powder from dogfish livers.

RESULTS

A series of experiments was undertaken to show that the dogfish enzyme, although lacking the capacity to associate to any appreciable extent at high concentrations, was truly analogous in

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other respects to glutamate dehydrogenases derived from other animal sources. Since such a similarity had already been established in the previous report (5) with the TPN^+s as coenzymes, it seemed worthwhile to appraise some of the enzyme's kinetic characteristics with the DPN^+s as coenzymes.

Fig. 1, a reciprocal plot of activity against DPN^+ concentration, reveals the activation at high concentrations of DPN^+ that has been found to be characteristic of the glutamate dehydrogenases described in the literature. The *solid line* of the graph has been calculated with the use of the equation proposed by Frieden (6) for a mechanism that involves the binding of excess DPN^+ to a site on the enzyme in addition to the active site. The DPN^+ bound in this way is held to be responsible for the activation that is observed.

$$v = \frac{V_1 + V_2 \frac{DPN^+}{K_2}}{1 + \frac{K_1}{DPN^+} + \frac{DPN^+}{K_2}} \quad (1)$$

The kinetic constants for DPN^+ in the presence of 0.05 M glutamate are as follows.

$K_1 = 1.2 \times 10^{-4}$ M

$V_1/E_0 = 5.72$ μmoles of DPN^+ per min per mg of enzyme

$K_2 = 2.4 \times 10^{-3}$ M

$V_2/E_0 = 10$ μmoles of DPN^+ per min per mg of enzyme

The determination of the K_m for glutamate is graphically depicted in Fig. 2. In the presence of 1.9×10^{-4} M DPN^+, the values for the kinetic constants for glutamate are

$K = 2.5 \times 10^{-3}$ M

$V_{max} = 6$ μmoles of DPN^+ per min per mg

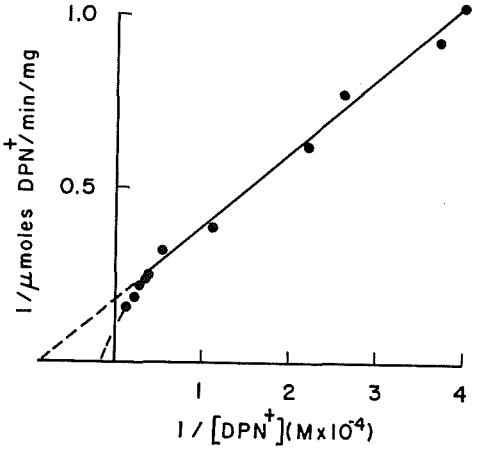

Fig. 1. Double reciprocal plot of velocity with respect to DPN^+ concentration. Experiments were performed in 0.01 M Tris-acetate buffer, pH 8.0, containing 10^{-5} M EDTA. The final concentration of glutamate was 0.05 M and the reactions were initiated by the addition of enzyme. Velocity represents the number of micromoles of DPN^+ per min per mg of enzyme. The *solid line* was calculated with the use of the equation

$$v = \frac{V_1 + V_2 \frac{DPN^+}{K_2}}{1 + \frac{K_1}{DPN^+} + \frac{DPN^+}{K_2}}$$

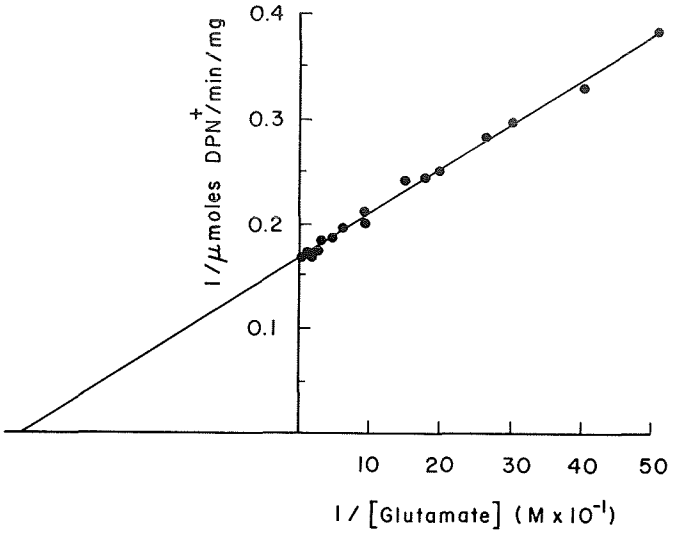

Fig. 2. Double reciprocal plot of activity with respect to glutamate concentration. The experiments were performed in 0.01 M Tris-acetate buffer, pH 8.0, containing 10^{-5} M EDTA. The final concentration of DPN^+ was 1.9×10^{-4} M and the reaction was initiated by the addition of enzyme.

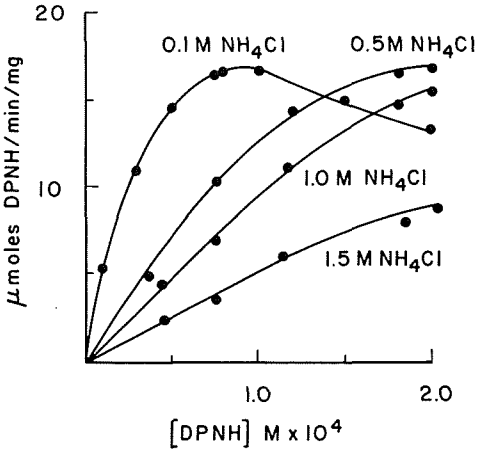

Fig. 3. The effect of increasing concentrations of ammonium chloride on DPNH inhibition of dogfish glutamate dehydrogenase. The experiments were performed in 0.01 M Tris-acetate buffer, pH 8.0, containing 10^{-5} M EDTA. The final concentration of α-ketoglutarate was 5×10^{-3} M. The concentrations of DPNH and ammonium chloride were as indicated. The reaction was initiated by the addition of the enzyme. The velocities represent the number of micromoles of DPNH oxidized per min per mg.

Evaluation of the kinetic constants for the reductive reaction was made more complicated by the preliminary observation that increasing quantities of ammonium chloride tended to reverse the inhibition normally encountered at high levels of DPNH. This is shown in Fig. 3 for dogfish glutamate dehydrogenase, although the initial observation had been made with the enzyme from chicken liver in a similar set of experiments. The data shown in Fig. 3 suggested that ammonium or chloride ion, or both, were inhibitory at low concentrations of DPNH but appeared to activate at high concentrations of DPNH. These possibilities prompted the comparison of ammonium chloride with ammonium acetate as substrates at varying DPNH concentrations. The results of such a study are presented in Fig. 4.

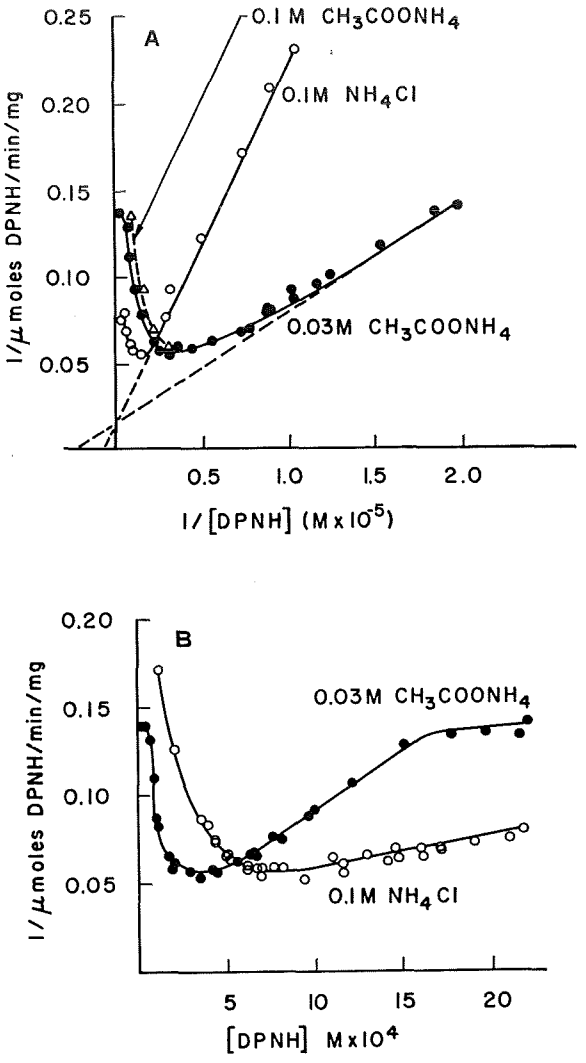

FIG. 4. *A*, double reciprocal plots of velocity at various DPNH concentrations in the presence of ammonium chloride and ammonium acetate. Reaction conditions were the same as described for previous figures. *B*, a replot of the data of *A* to enable a clearer comparison of these substrates at high DPNH concentrations. The *solid lines* in both *A* and *B* have been calculated with the equation

$$v = \frac{V}{1 + \frac{K_1}{\text{DPNH}} + \frac{\text{DPNH}}{K_2}}$$

See text for a consideration of the lack of conformity with the equation at "high" DPNH concentrations.

The *solid lines* have been calculated from the same equation as that used for the calculation of the DPN^+ constants. In this instance, however, the equation has been simplified by the assumption that the binding of a 2nd molecule at the inhibitory site on the enzyme leads to an essentially inactive complex; that is, V_2 is taken to be 0 in analogy with the study of the frog liver enzyme (3). Under these circumstances the equation was simplified to

$$v = \frac{V}{1 + \frac{K_1}{\text{DPNH}} + \frac{\text{DPNH}}{K_2}} \qquad (2)$$

From the mechanism proposed and the derivation of the equation that follows from it, it can be seen that K_1 represents a Briggs-Haldane constant that includes the dissociation constant for the active complex, as well as the kinetic constant for its breakdown to product. K_2, on the other hand, represents solely the dissociation constant for the inactive enzyme-coenzyme complex. Thus the value of K_1 determined in this fashion provides no insight into the affinity of the enzyme for the coenzyme at the active site and actually appears to exceed the value obtained for K_2. When ammonium acetate was used as the substrate, these constants had the following values.

$$K_1 = 4.5 \times 10^{-5} \text{ M}; \qquad K_2 = 2 \times 10^{-5} \text{ M}$$

When ammonium chloride was used as the substrate, the kinetic constants for DPNH were somewhat different.

$$K_1 = 15 \times 10^{-5} \text{ M}; \qquad K_2 = 5.5 \times 10^{-5} \text{ M}$$

Interestingly enough, in either case the maximum velocity remained constant at 71.5 μmoles of DPNH oxidized per min per mg of enzyme.

In order to evaluate more clearly the effects of high DPNH concentration the data of Fig. 4*A* have been replotted in Fig. 4*B* as the reciprocal velocity against substrate concentration. If Equation 2 is valid, the term K_1/DPNH would be expected to become less significant with increasing substrate concentration, and the data, as shown, indicate this to be the case. The effect is more pronounced with ammonium acetate than it is with ammonium chloride. Moreover, it would appear that there is a limit to the extent of DPNH inhibition, although this is not apparent when the chloride ion is present. Thus it may not be at all proper to assume that $V_2/E_0 = 0$, as has been done, but rather some very small finite value. In essence, K_2 would still represent a dissociation constant. It is also clear from these results that an increase in the concentration of ammonium acetate (0.03 to 0.1 M) has little effect, whereas the presence of the chloride salt has a marked effect on the amount of DPNH required to achieve peak inhibition.

Since the data shown in Fig. 4 had been obtained at 0.03 M ammonium acetate and at 0.1 M ammonium chloride, it was necessary to show that the cardinal difference between these two, in terms of DPNH kinetics, was not attributable to the increased ammonium ion concentration alone. To this end a range of ammonium acetate concentrations has been studied at fixed levels of DPNH and α-ketoglutarate, 1.2×10^{-4} M and 5×10^{-3} M, respectively, in the presence and absence of added salt. As is shown in Fig. 4*A*, increasing the concentration of ammonium acetate from 0.03 M to 0.1 M very definitely did not overcome the inhibition caused by excess DPNH. The maximum velocity achieved in the experiments, represented by Fig. 5, was limited by the inhibitory amount of DPNH present (*cf.* Fig. 2). Moreover, the ammonium ion itself produced some substrate inhibition at concentrations above 0.02 M. Inasmuch as added sodium acetate, up to 0.2 M, simply decreased the reaction velocity further, the influence of ammonium chloride as substrate on the kinetic constants for DPNH must be directly attributable to the presence of the chloride anion. Similar results have been obtained with chicken and beef liver glutamate dehydrogenases, an indication that this behavior is not peculiar to the dogfish enzyme. It is also apparent from Fig. 5 that the addition of salt has not only overcome the inhibition by excess DPNH but also that produced by excess ammonium ion. Again,

the maximum velocity has remained unaffected by added salt, although the apparent K_m for the substrate has been changed. It is of interest that, in the presence of added salt, the apparent K_m for ammonium acetate approaches 0.08 M, the value reported in the previous paper when TPNH was used as the coenzyme for the reaction.

The evaluation of the Michaelis constant for α-ketoglutarate is made difficult because of the inhibition encountered at high levels of ammonium acetate and DPNH. As shown by the data in Fig. 6, at low levels of ammonium acetate and at a fixed inhibitory concentration of DPNH, although the maximum velocity was limited by the DPNH present, the Michaelis constant for α-ketoglutarate was very low (5×10^{-4} M). At a higher concentration (0.1 M), ammonium acetate inhibition caused a marked increase in the α-ketoglutarate constant. When, however, the same concentration (0.1 M) of ammonium chloride was used, it became apparent that the inhibition by both ammonium ion and DPNH had been reversed. Under these circumstances the K_m for α-ketoglutarate was found to be the same as that observed when the reaction had been studied with TPNH (4.5×10^{-3} M). The data presented in Fig. 7 were obtained at a low, fixed concentration of ammonium acetate (0.03 M) and at several constant levels of DPNH. At this concentration of ammonium acetate, the Michaelis constants for α-ketoglutarate and DPNH appear to be independent of each other. This same phenomenon occurred with beef liver glutamate dehydrogenase (7) when TPNH had been used as the coenzyme for the reaction. On the assumption that the mechanism proposed by Frieden is correct, it would follow that the ammonium ion is also the second substrate in the sequential order of addition of substrates to the enzymes when DPNH is used.

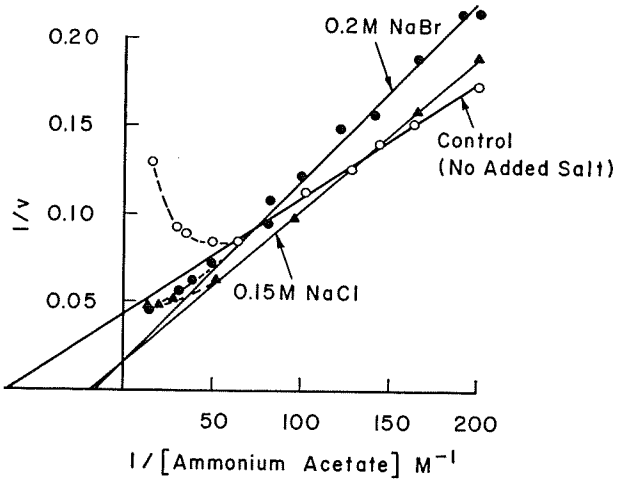

FIG. 5. The effect of added salt on the K_m for ammonium acetate. The final concentration of DPNH was 1.2×10^{-4} M. All other conditions were as previously described, except for the concentrations of added salts as indicated: ○, no added salt; ●, +0.2 M NaBr; ▲, +0.15 M NaCl.

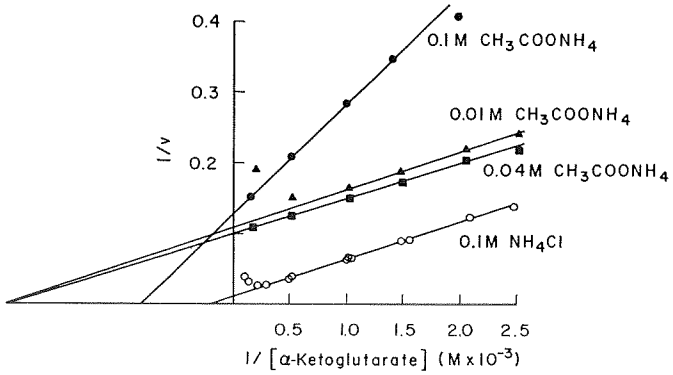

FIG. 6. Comparison of the effects of ammonium acetate and ammonium chloride on kinetics of α-ketoglutarate (at high DPNH concentration, 1.2×10^{-4} M), 0.01 M Tris-acetate (pH 8), 10^{-5} M EDTA.

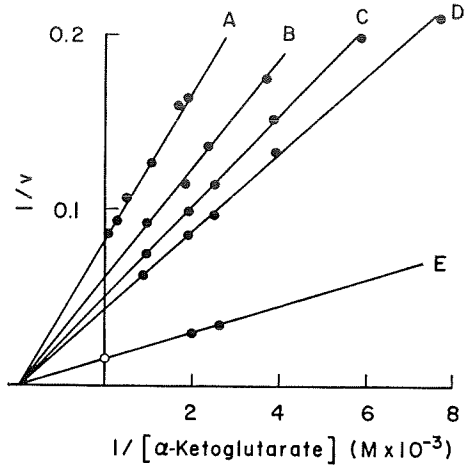

FIG. 7. Double reciprocal plots of α-ketoglutarate concentration with respect to activity at a fixed, low concentration of ammonium acetate (0.03 M) and at various constant levels of DPNH: *A*, 1×10^{-5} M; *B*, 1.5×10^{-5} M; *C*, 2×10^{-5} M; *D*, 2.5×10^{-5} M; *E*, the extrapolated line for an infinite concentration of DPNH. Other conditions were as previously described.

TABLE I

Summary of kinetic constants for dogfish glutamate dehydrogenase with DPN^+

	Constants
$K_{glutamate}$	2.5×10^{-3} M
K_1 DPN^+	1.2×10^{-3} M
K_2 DPN^+	2.4×10^{-3} M
V_1E_0	32 moles of DPN^+ per mole of enzyme per sec
V_2E_0	57 moles of DPN^+ per mole of enzyme per sec

TABLE II

Summary of kinetic constants for dogfish glutamate dehydrogenase with DPNH

	Constants	
	Ammonium chloride	Ammonium acetate
K_{NH4}	8×10^{-2} M	1.4×10^{-2} M
$K_{ketoglutarate}$	4.5×10^{-3} M	5.0×10^{-4} M
K_1DPNH	15×10^{-5} M	4.5×10^{-5} M
K_2DPNH	5.5×10^{-5} M	2.0×10^{-5} M
V_f/E_0		400 moles of DPNH per mole of enzyme per sec

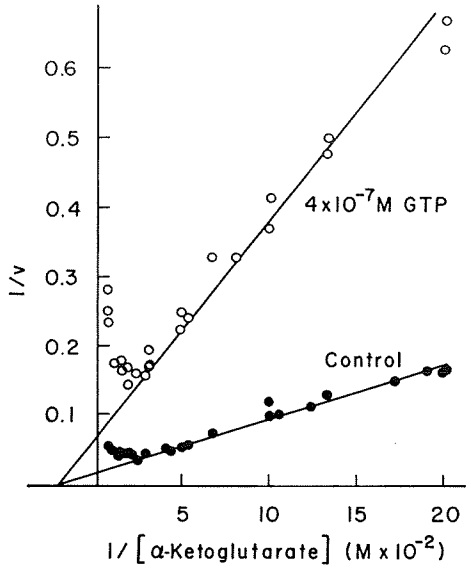

FIG. 8. Noncompetitive nature of the inhibition by GTP. Experiments were performed in 0.01 M Tris-acetate buffer, pH 8.0, containing 10^{-5} M EDTA. The final concentration of DPNH was 1.2×10^{-4} M, of ammonium chloride was 0.1 M, and of α-ketoglutarate and GTP as indicated.

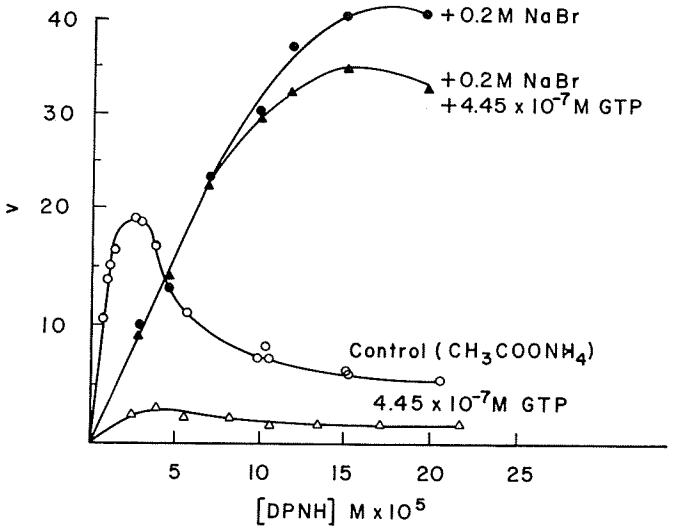

FIG. 9. Effect of added salt on GTP and DPNH inhibition of dogfish glutamate dehydrogenase with ammonium acetate as substrate. Experiments were performed in 0.01 M Tris-acetate buffer, pH 8.0, containing 10^{-5} M EDTA. The final concentration of α-ketoglutarate was 5×10^{-3} M, of ammonium acetate was 0.1 M, and of other reactants as indicated. The velocities reported represent the number of micromoles of DPNH per min per mg of enzyme.

The kinetic constants that have been evaluated to this point are summarized in Tables I and II. They clearly show that there is a considerable difference between the constants, depending on the presence or absence of the chloride anion.

In analogy with the other glutamate dehydrogenases that have been studied, Fig. 8 reveals that GTP is a noncompetitive inhibitor with respect to α-ketoglutarate in the dogfish system. It thus appears that GTP must bind to the enzyme at a site other than the active site. The effects of added GTP as a function of increasing concentrations of DPNH, with ammonium acetate and ammonium chloride as substrates, respectively, are shown in Figs. 9 and 10; GTP was a more effective inhibitor in the presence of ammonium acetate than when ammonium chloride was used. In fact, twice as much GTP was needed to produce the same degree of inhibition with ammonium chloride as substrate. The chloride anion as part of the added substrate partially relieved the inhibition caused by GTP. This can be seen more clearly upon the addition of more salt which, in both instances, clearly reversed the inhibition by GTP, as well as that caused by increasing DPNH concentrations. It was also apparent from these data that at low concentrations of DPNH the addition of salt caused some inhibition.

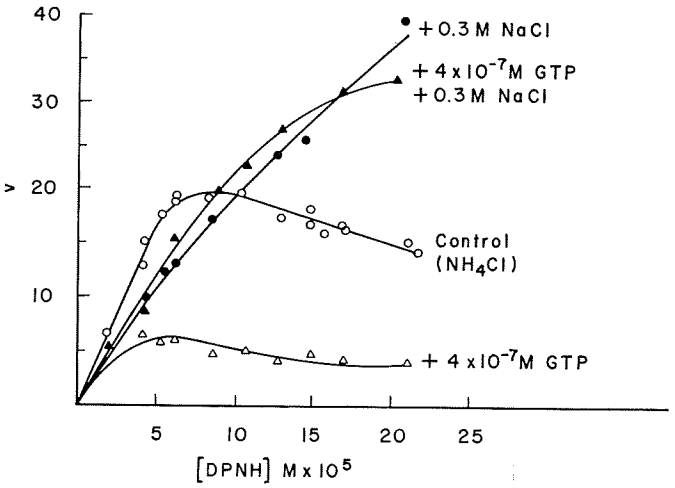

FIG. 10. Effect of added salt on GTP and DPNH inhibition of dogfish glutamate dehydrogenase with ammonium chloride as substrate. The same conditions were followed as described in the legend for Fig. 9, except for the use of ammonium chloride as substrate.

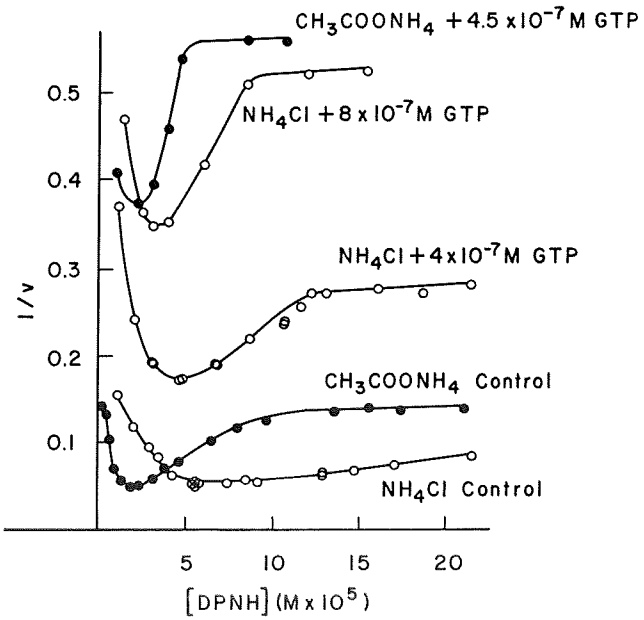

FIG. 11. Reciprocal velocity with respect to increasing DPNH concentration in the presence of GTP, illustrating the difference between ammonium acetate and ammonium chloride as substrates. The experimental conditions were the same as those described for Figs. 9 and 10.

When some of the data of Figs. 9 and 10 were replotted as reciprocal velocity against increasing DPNH concentrations, as shown in Fig. 11, one can see that the effect of GTP, far more pronounced when ammonium acetate is the substrate, is partly analogous to that shown in Fig. 4*B*, when the ammonium acetate concentration was increased from 0.03 M to 0.1 M; that is to say, in the presence of GTP, the inhibitory limit of substrate DPNH is reached at a lower concentration. Moreover, the limit of DPNH inhibition occurred at a lower concentration of this substrate as the GTP concentration increased, even in the presence of ammonium chloride.

The effect of ADP on the kinetics for DPNH is illustrated in Fig. 12. The activation that has been observed with glutamate dehydrogenases from several sources is apparently true for the dogfish enzyme as well. The *dashed line* represents an extrapolation from concentrations of DPNH considerably lower than those encompassed by the range shown on the *abscissa* of the graph. At higher concentrations, as has been shown above, DPNH is inhibitory. At relatively low DPNH concentrations the observed activity with ADP, although greater, parallels the extrapolated control values, so that it can be said that ADP, at these concentrations, exhibits "uncompetitive" kinetic behavior with respect to DPNH, as observed with beef liver glutamate dehydrogenase by Frieden (1). At higher DPNH concentrations the activation induced by the presence of ADP was largely overcome, and the velocity rapidly fell because of DPNH inhibition. Again, added salt clearly reversed the activation by ADP, but salt alone did not alter the extrapolated maximum velocity.

To appraise the influence of different anions on the reductive amination of α-ketoglutarate, the experiments represented by Fig. 13 were performed. They were done at inhibitory concentrations of both DPNH (1.2×10^{-4} M) and ammonium acetate (0.1 M) in order to magnify the difference among the anions. The order of effectiveness of these anions in relieving the inhibition was $ClO_4^- > SCN^- > I^- > Br^- > Cl^-$. At the present

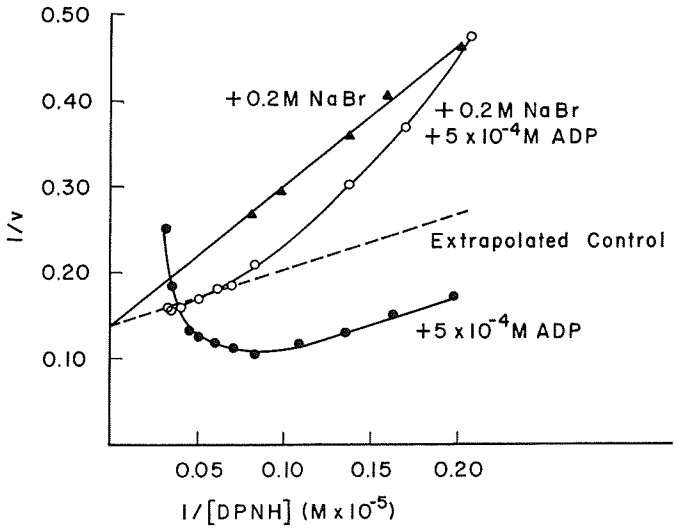

FIG. 12. Comparison of the effects of added salt or ADP or both on the kinetics of DPNH turnover by dogfish glutamate dehydrogenase. Except where indicated, the experimental conditions were as previously described. The extrapolated control line was derived from data obtained at considerably lower concentrations of DPNH. In the concentration range encompassed by the *abscissa* values of this figure there would be marked inhibition by DPNH.

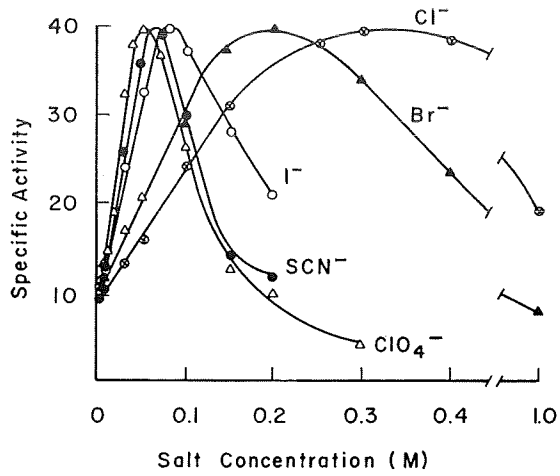

FIG. 13. Effect of increasing concentrations of various anions on reductive reaction of dogfish glutamate dehydrogenase.

α-Ketoglutarate + NH_4^+ + DPNH $\rightarrow$ glutamate + DPN^+ + H_2O

The reaction mixture consisted of 1×10^{-4} M DPNH, 0.1 M ammonium acetate, 0.01 M α-ketoglutarate, and salts as indicated, in 0.01 M Tris-acetate buffer, pH 8.0, containing 10^{-5} M EDTA. The reaction was initiated by the addition of enzyme.

time, it appears that fluoride is considerably less effective than chloride. The same order of effectiveness of the various anions has been found for the enzymes from beef and chicken livers.

DISCUSSION

The dogfish glutamate dehydrogenase is anomalous among the glutamate dehydrogenases of animal origin that have been studied because of its demonstrable inability to associate or dissociate to any significant extent in ultracentrifugal studies performed at high and low concentrations in the presence of added modifiers. However, its kinetic behavior under all the conditions investigated has been strikingly similar to that of other animal glutamate dehydrogenases. This in no way denies the invocation of conformational changes to explain the altered enzymatic activity in the presence of various modifiers. However, it does indicate that conformational changes of the dogfish enzyme, if they do occur, are not reflected in changes detectable by sedimentation studies, and also that aggregation is not essential for the action of the nucleotides.

It has become increasingly clear during the course of these studies that the use of ammonium chloride as substrate at the concentrations commonly used (0.1 M or more) distorts the kinetic picture. This is of some importance since ammonium chloride has been the substrate of choice in most studies of glutamate dehydrogenases, whatever their source. Olson and Anfinsen (8) in their work on beef liver glutamate dehydrogenase noted the inhibitory effect of relatively high (1.5 M) concentrations of ammonium chloride. They were inclined to attribute the inactivation of the enzyme to the ionic strength of the solution. Snoke (9) in his study of chicken liver glutamate dehydrogenase observed inhibition of the oxidative deamination reaction by several salts. He showed that it occurred at relatively low levels of salts (0.1 to 0.3 M) and was not related to ionic strength.

A recent study (10) comparing the activities of glutamate dehydrogenases from lobster muscle, crab muscle, and beef

liver, in the presence of various salts, reported a marked activation of reductive animation by the beef liver enzyme upon the addition of chloride or sulfate ions and inhibition by added nitrate. The same effect is observed with the chicken and dogfish liver glutamate dehydrogenases. However, from the data presented here, it is apparent that added salt does not alter the maximum velocity of the reaction (DPNH $\rightarrow$ DPN^+). Therefore, it may be misleading to speak of "activation" under circumstances in which the enzyme is inhibited by both excess DPNH and ammonium acetate. Moreover, as has been shown in Figs. 10 and 11, at low, noninhibitory levels of DPNH, added salt causes a substantial reduction of activity, which is particularly noticeable when ammonium acetate is used as substrate.

The presence of salt exerts a stabilizing effect on solutions of beef liver glutamate dehydrogenase (11). In addition, it clearly protects the dogfish enzyme against the influence of the three modifiers examined in this study, excess DPNH, ADP, and GTP. These observations, plus the ready reversibility of the salt effect and the order of effectiveness of the anions, which is directly analogous to that observed by Robinson and Jencks (12) for a model peptide, strongly suggest that the effect of the salt is exerted directly on the protein.

The concentrations of salts used are not incompatible with physiological conditions. Thus these findings may be of some significance *in vivo*.

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